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trk C Receptor Signaling Regulates Cardiac Myocyte Proliferation during Early Heart Development *in Vivo*

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Neurotrophin-3 (NT-3) is a member of the neurotrophin family of growth factors, best characterized by its survival- and differentiation-inducing effects on developing neurons bearing the trk C receptor tyrosine kinase. Through analysis of NT-3 and trk C gene-targeted mice we have identified NT-3 as critically regulating cardiac septation, valvulogenesis, and conotruncal formation. Although these defects could reflect cardiac neural crest dysfunction, the expression of NT-3 and trk C by cardiac myocytes prior to neural crest migration prompted analysis of cell-autonomous actions of NT-3 on cardiac myocytes. Retroviral-mediated overexpression of truncated trk C receptor lacking kinase activity was used to inhibit activation of trk C by endogenous NT-3, during early heart development *in ovo*. During the first week of chicken development, expression of truncated trk C reduced myocyte clone size by more than 60% of control clones. Direct mitogenic actions of NT-3 on embryonic cardiac myocytes were demonstrated by analysis of BrdU incorporation or PCNA immunoreactivity in control and truncated trk C-expressing clones. Inhibition of trk C signaling reduced cardiac myocyte proliferation during the first week of development, but had no effect at later times. These studies demonstrate that endogenous NT-3:trk C signaling regulates cardiac myocyte proliferation during cardiac looping and the establishment of ventricular trabeculation but that myocyte proliferation becomes NT-3 independent during the second week of embryogenesis. © 2000 Academic Press

Key Words: **cardiac myocyte proliferation; neurotrophin-3; trk C receptor; cardiogenesis.**

INTRODUCTION

The development of the functional heart depends upon the coordinated growth, differentiation, migration, and apoptosis of cell populations of diverse embryological origins. The cardiac myocyte plays a central role in this process, beginning with commitment to a myocyte lineage and acquisition of a rhythmically contractile phenotype. In the well-studied chick model, cardiac myocytes actively proliferate in the ventricular walls early in development during ventricular trabeculation, forming a contractile lattice within the ventricular chambers. Subsequently, the outer myocardial layer thickens, giving rise to the compact zone, which generates the outer wall of the ventricular chambers. In later embryogenesis, the mitotic activity of cardiac myocytes slows and is lost soon after birth (Clark and Fischman, 1983).

Few growth factors which directly regulate cardiac myocyte proliferation have been identified despite the identification of gene-targeted mouse models with abnormalities in ventricular morphogenesis and *in vitro* studies. The neuregulins and cardiotrophin-1 were first hypothesized to regulate myocyte proliferation based on the prominent ventricular phenotypes of gene-targeted mice (Gassmann *et al.*, 1995; Meyer and Birchmeier, 1995; Wollert and Chien, 1997). However, these factors demonstrate primarily hypertrophic and survival activities on cultured neonatal rodent cardiac myocytes (Ford *et al.*, 1999; Latchman, 2000; Zhao *et al.*, 1998). More recently, retinoic acid and erythropoietin have been postulated to act indirectly to promote myocyte proliferation (Tran and Sucov, 1998; Wu *et al.*, 1999). The

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best characterized mitogenic factors, however, are members of the fibroblast growth factor (FGF) family, specifically FGF-1, -2, and -4. The FGFs induce cardiac myocyte proliferation *in vitro* (Zhu *et al.,* 1996) and inhibition of FGF receptor signaling *in vivo* decreases myocyte proliferation during early embryogenesis in the chick (Mima *et al.,* 1995).

Our identification of abnormal cardiac morphogenesis leading to the early postnatal lethality in mice with decreased neurotrophin-3 expression (Donovan *et al.,* 1996) prompted an evaluation of direct actions of this growth factor on embryonic cardiac myocytes. The neurotrophins are a family of polypeptide growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4/5 (NT-3 and NT-4/5), which have been best characterized for their critical roles in neuronal differentiation and survival (Snider, 1994). The neurotrophins mediate their actions by binding to the trk family of receptor tyrosine kinases, with NGF binding to trk A, BDNF and NT-4/5 to trk B, and NT-3 to trk C. NT-3 binding results in trk C kinase activation, leading to the recruitment and phosphorylation of signaling proteins which regulate neuronal proliferation, differentiation, and survival. In addition, alternative splicing of the trk C gene can generate truncated trk C isoforms which lack the kinase domain (Garner and Large, 1994; Tsoulfas *et al.,* 1993; Valenzuela *et al.,* 1993). These noncatalytic receptors, which are well conserved among species, inhibit signaling by catalytic receptors when both catalytic and truncated isoforms are coexpressed (Das *et al.,* 2000). Indeed, overexpression of truncated trk C in a transgenic mouse model leads to a phenotype similar to that observed in trk C or NT-3 null mutant animals, suggesting that the truncated trk C receptors negatively modulate signaling by kinase active trk C (Palko *et al.,* 1999).

Although trk C is highly expressed by neurons, it is also expressed in muscle, lung, kidney, heart, and vascular smooth muscle cells (Donovan *et al.,* 1995; Tessarollo *et al.,* 1993) where its biological actions are unclear. We have recently localized trk C expression to mouse embryonic cardiac myocytes and have demonstrated that NT-3 and trk C critically regulate cardiac morphogenesis, with NT-3 (−/−) and trk C (−/−) animals exhibiting ventricular septal defects, delay in conotruncal septation and rotation, and valvular defects (Donovan *et al.,* 1996; Tessarollo *et al.,* 1997). Since the septation of the outflow tracts and valvular morphogenesis are influenced, in part, by cardiac neural crest, this constellation of defects might reflect impaired neural crest migration, proliferation, or survival. However, the early expression of trk C by cardiac myocytes prompted investigation of potential direct actions of NT-3 in cardiac myocyte development. Thus, we have utilized retroviral gene delivery to alter NT-3 signaling by cardiac myocytes during the first week of avian development. Replication-deficient recombinant retrovirus encoding the chicken truncated trk C receptor was delivered to the developing heart to inhibit NT-3-dependent trk C activation. Using clonal analysis, these *in vivo* studies have allowed us to assess the stage-specific effects of the impaired NT-3 signaling on cardiac myocyte proliferation, migration, and survival.

MATERIAL AND METHODS

Retroviral vectors. The pCXIZ retrovirus is a replication-defective variant based on the spleen necrosis virus (Mikawa and Fischman, 1992), which utilizes an internal ribosome entry sequence (IRES) genome upstream of the bacterial β-galactosidase (β-gal) gene to identify cells expressing viral transgenes. The cDNA for chicken truncated trk C corresponds to the kinase-deleted isoform (Garner and Large, 1994). Sequences from −15 to 1598, which includes the entire cDNA and 15 and 8 bases upstream and downstream, respectively, were subcloned upstream of the IRES, and the resulting vector was designated trunc C. Following cotransfection of the packaging line ND17.2G with the trunc C vector and pMEX neo, stable clones were isolated following selection in G418 as described (Mikawa and Fischman, 1992). Cells from single colonies were subjected to cloning by limiting dilution for three successive rounds to establish clonal lines with titers of $4-8 \times 10^4$ virions/ml. Infection of chick embryo fibroblasts was performed to document viral titer and to confirm the absence of helper virus (Mikawa *et al.,* 1991). The retrovirus CXL (Mikawa and Fischman, 1992), expressing only cytoplasmically localized β-gal, was used as a control.

In ovo infection. Concentrated viral solutions ($>10^6$ virions/ml) of 5–10 nl, containing Polybrene (100 μg/ml), were pressure injected into the myocardial wall of chick hearts at embryonic day 2, 3, or 5 (E2, E3, E5), using a Picospritzer II (General Valve, NJ) and a Leitz micromanipulator (No. 031626). Eggs were resealed with Parafilm and embryos were returned to a humidified incubator at 37.5°C for further development. Selected embryos were pulse-labeled with BrdU by injecting BrdU (Amersham), 75 μg for E5 and E6 or 150 μg for E9 embryos, onto the amniotic membrane for 5 h prior to embryo harvest.

Morphologic analysis. Infected embryos were fixed by immersion overnight at 4°C in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and processed for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Gold Biotechnology, Inc.) histochemistry, in whole mount as described (Mikawa *et al.,* 1992a). Hearts exhibiting β-gal-positive colonies were embedded in Paraplast (Oxford Labware), serially sectioned at 10 μm thickness, and examined following counterstaining with eosin.

Analysis of truncated trk C expression. ND17.2G clones stably transfected with the trunc C pCXIZ vector were lysed in RIPA buffer containing the protease inhibitors as described (Mahadeo *et al.,* 1994). Lysates containing equivalent amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose. Western blot analysis was performed using a polyclonal antisera specific for a unique sequence in the cytoplasmic tail of the truncated trk C isoform (Donovan *et al.,* 1995; Tsoulfas *et al.,* 1993).

To confirm NT-3 binding to truncated trk C, NT-3 (human recombinant; Promega, Madison, WI) was radioiodinated using lactoperoxidase as described (Hempstead *et al.,* 1989) to a specific activity of 2500 cpm/fmol. The ND17.2G expressing truncated trk C (trunc C) or parental ND17.2G cells (1×10^6 cells/ml) were incubated with ^{125}I-NT-3 (50 ng/ml) for 2 h at 4°C as described (Kaplan *et al.,* 1991). Bound NT-3 was crosslinked to cells by exposure to 50 mM *N*-hydroxysuccinimidyl-4-azido benzoate (HSAB) (Pierce, Rockford, IL) for 10 min with photoactivation using

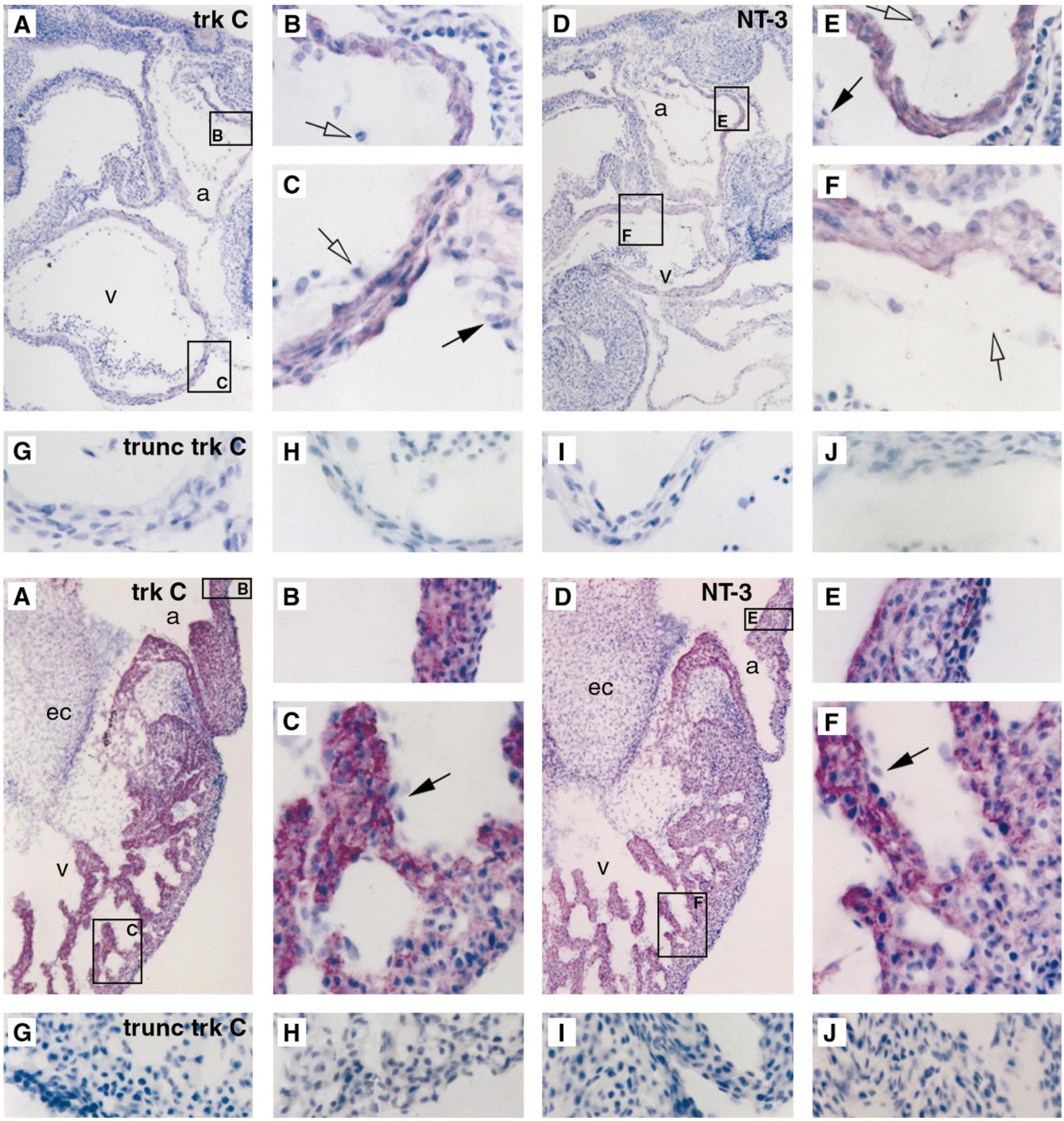


FIG. 1. Expression of NT-3, trk C, and truncated trk C in the avian heart during looping (HH stage 17). Immunohistochemical detection of NT-3, full-length trk C, and truncated trk C expression patterns in sections of chick heart at E 2.5. Heart sections were incubated with anti-full-length trk C antiserum (A–C), anti-NT-3 antiserum (D–F), anti-truncated trk C antiserum (G), or preimmune serum as a control for the truncated trk C antisera (H), preimmune serum as the trk C antiserum control (I), or anti-NT-3 preincubated with the immunizing peptide (J). The positions of higher magnification views (B, C, E, F) are indicated on lower magnification views (A, D). Immunoreactive products were visualized with VIP as the chromogenic substrate (red reaction product). a, atrium; v, ventricle; closed arrow, epicardium; open arrow, endocardium. (A and D, original magnification 40×; B, E, G, H, I, and J, 400×; C, F, 600×).

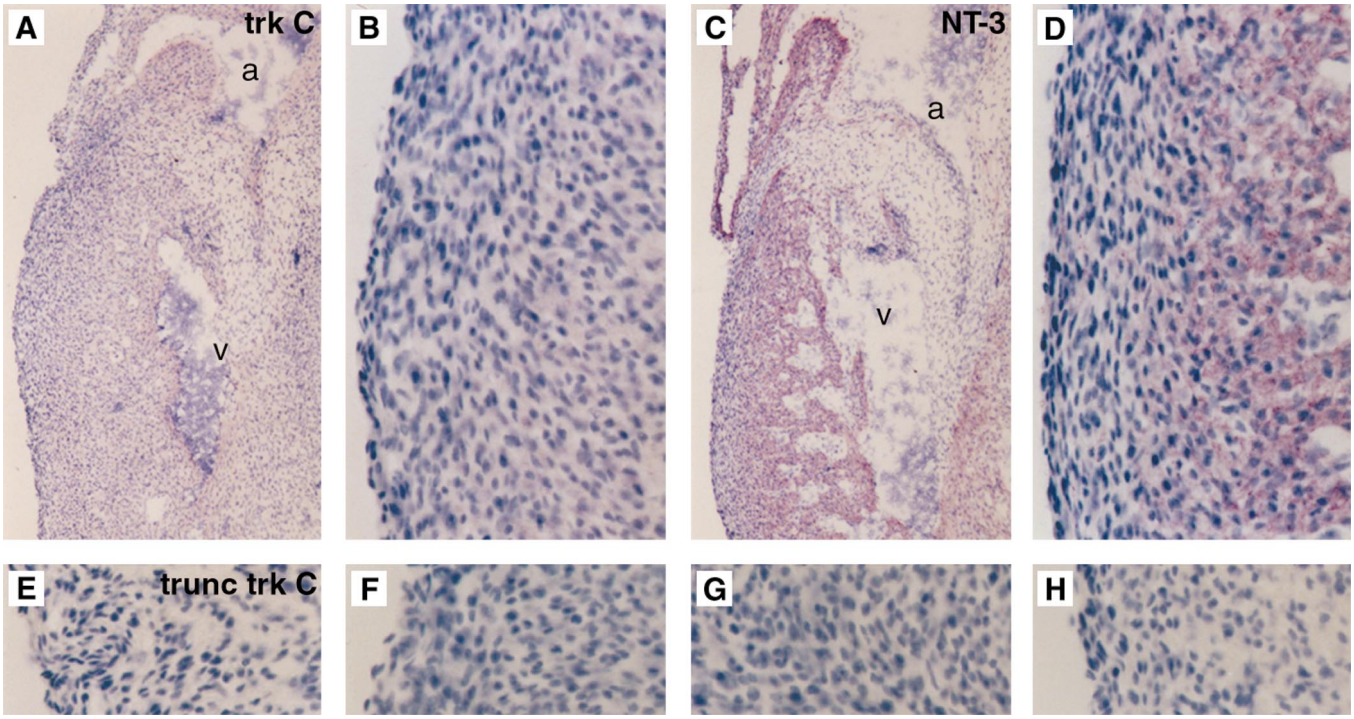


FIG. 3. Expression of NT-3, trk C, and truncated trk C in the avian heart at HH stage 35 (E9). Immunohistochemical detection of NT-3, full-length trk C, and truncated trk C expression patterns in sections of chick heart at E9. Heart sections were incubated with anti-full-length trk C antiserum (A, B), anti-NT-3 antiserum (C, D), anti-truncated trk C antiserum (E), or preimmune serum as a control for the truncated trk C antiserum (F), preimmune serum as the trk C antiserum control (G), or anti-NT-3 preincubated with the immunizing peptide (H). Immunoreactive products were visualized with VIP as the chromogenic substrate (red reaction product). a, atrium; v, ventricle. (A and C, original magnification 40×; B, D, E, F, G, and H, 400×).

a 365-nm UV lamp. Cells were washed twice in 50 mM lysine in PBS to quench the HSAB and lysed in RIPA buffer containing protease inhibitors. Proteins were separated by SDS–PAGE and autoradiography was performed as described (Hempstead *et al.*, 1991).

Immunohistochemical analysis. Immunohistochemistry to evaluate expression of NT-3, kinase-active trk C, or truncated trk C in uninfected chick embryos was performed using dissected tissues from embryos at E3, E6, and E9, which were fixed for 2 h in 0.1% paraformaldehyde in PBS. Cryoprotected tissues were sectioned at 10 μm and incubated with rabbit polyclonal antiserum specific for NT-3 (1:200) (sc-547; Santa Cruz), rabbit polyclonal antiserum specific for kinase active trk C (Donovan *et al.*, 1995) (generated using the peptide LVDGQPRQAKGELGL, used at 1:200), or rabbit polyclonal antiserum specific for truncated trk C (Donovan *et al.*, 1995) (generated using the peptide LNPISLPGHSKPLNQG, a unique sequence in the cytoplasmic tail of the truncated trk C isoform, at 1:200 dilution). Following washing, sections were incubated with biotinylated anti-rabbit antibody and immunoreactivity was detected using VIP substrate (Vector Laboratories) after ABC signal amplification (Vector Laboratories). Preincubation of antiserum with the immunizing peptide was used to confirm antibody specificity. In addition, these antisera have been utilized on NT-3 or trk C gene-targeted mouse tissues and a lack of immunoreactivity has been confirmed.

Immunohistochemistry was performed on tissue sections from infected embryos using the polyclonal antiserum recognizing truncated trk C (1:500) (Donovan *et al.*, 1995) and a monoclonal

FIG. 2. Expression of NT-3, trk C, and truncated trk C in the avian heart during septation and trabeculation at HH stage 28 (E6). Immunohistochemical detection of NT-3, full-length trk C, and truncated trk C expression patterns in sections of chick heart at E6. Heart sections were incubated with anti-full-length trk C antiserum (A–C), anti-NT-3 (D–F), anti-truncated trk C antiserum (G), or preimmune serum as a control for the truncated trk C antiserum (H), preimmune serum as the trk C antiserum control (I), or anti-NT-3 preincubated with the immunizing peptide (J). The positions of higher magnification views (B, C, E, F) are indicated on lower magnification views (A, D). Immunoreactive products were visualized with VIP as the chromogenic substrate (red reaction product). a, atrium; v, ventricle; closed arrow, endocardium. (A and D, original Magnification 40×; B, E, G, H, I, and J, 400×; C, F, 600×).

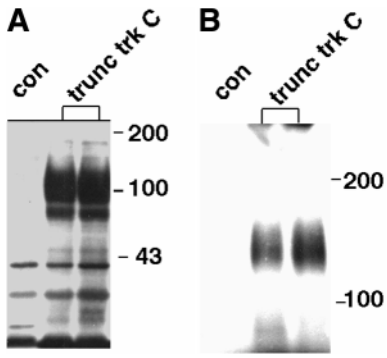


FIG. 4. Expression of truncated trk C by the transfected packaging cell line. (A) Western blots of cell lysates of parental (con) or two independent truncated trk C (trunc trk C)-expressing packaging cell lines were probed with truncated trk C antiserum. Results obtained using two independent lines stably expressing truncated trk C are shown. Expression of the truncated trk C receptor was observed at 110 kDa. (B) NT-3 binding to truncated trk C was detected by crosslinking. Two independent lines stably expressing truncated trk C and the control parent cell line were incubated with ^{125}I-NT-3 following which bound NT-3 was crosslinked to cells using HSAB. Following detergent lysis, proteins were separated by SDS–PAGE and autoradiography was performed. The molecular size of the 120-kDa crosslinked species is consistent with the predicted molecular weight of the NT-3 and truncated trk C product (13 plus 110 kDa).

antiserum to β-galactosidase (1:500; Sigma Chemical Co., St. Louis, MO). Embryonic chick tissue was fixed in 2% paraformaldehyde in PBS and cryoprotected prior to frozen sectioning at 10 μm. Sections were blocked with 0.1% Triton X-100 in 5% normal goat serum in PBS prior to antibody addition. Following washing, sections were incubated with fluorescein-labeled goat anti-mouse or Texas red-labeled goat anti-rabbit antisera (each at 1:200), prior to washing and analysis using a Nikon epifluorescence microscope.

BrdU histochemistry was performed on paraffin-embedded sections treated with 0.1% hydrogen peroxide in methanol (−20°C), permeabilized with proteinase K (10 μg/ml), treated with 2 N HCl, and neutralized in 0.1 M Borax, pH 9.6, as per the commercial protocol. Sections were incubated with the monoclonal α-BrdU antiserum overnight at 4°C (1:500) (Dako A/S Denmark). Following washing, immune complexes were visualized using biotinylated anti-mouse IgG at 1:200 (Vector Laboratories) and the Vectastain Elite ABC Kit followed by the VIP substrate (Vector Laboratories).

TUNEL procedure was performed as per the manufacturer's recommendation (Roche Molecular Biochemicals) using frozen sections at 10 μm. Double-immunofluorescence detection of TUNEL and β-gal reactivity was visualized using a Zeiss Axioskop microscope.

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was performed on embryonic hearts treated with X-gal prior to paraffin embedding. Deparaffinized sections were treated with proteinase K (10 μg/ml) for 15 min followed by treatment with Target Unmasking Fluid (Signet) as per the manufacturer's protocol, prior to incubation with the monoclonal anti-PCNA antibody (Dako) at 1:500. After washing, immune complexes were visualized using biotinylated anti-mouse 1gG at 1:200 (Vector Laboratories) and the Vectastain Elite ABC Kit, followed by the VIP substrate (Vector Laboratories).

RESULTS

NT-3 and Kinase-Active Trk C Are Coexpressed by Cardiac Myocytes

To identify potential actions of NT-3 on cardiac morphogenesis, the expression of NT-3 and trk C in the developing heart was examined by immunohistochemical localization from HH stage 17 to 35. This time frame was chosen as it encompasses many of the major morphogenetic events, with the completion of cardiac looping (at HH 17, or approximately E2.5), the establishment of ventricular trabeculae (from HH stage 18 to stage 30), and cardiac septation (at HH 23–35) (Sissman, 1970). At HH stage 17, both kinase-active trk C and NT-3 are expressed by cardiac myocytes within regions of the looping heart which will develop into atria or ventricles (Figs. 1A–1F). Coexpression of ligand and catalytically active receptor is most prominent in embryonic myocytes, with little immunoreactivity of epicardial and endocardial cells (Figs. 1B, 1C, 1E, and 1F). Truncated trk C expression is not detectable in the developing heart at this stage (Fig. 1G).

In ventricles with a well-established trabecular lattice (HH 28, E6), expression of both NT-3 and trk C is regionally localized to myocytes within the trabeculations, whereas expression by subepicardial myocytes is diminished (Figs. 2A–2F). Myocytes within all four chambers of the heart exhibited similar levels of expression of NT-3 and trk C (Figs. 2A and 2D and data not shown). NT-3 and kinase-active trk C, however, are not expressed by cells within the endocardial cushions or by the endocardium (Figs. 2A, 2C, 2D, and 2F). No truncated trk C expression is noted in the heart at E6 (Fig. 2G). By E9 (HH stage 35), when the ventricular wall consists of a well-defined proliferating compact zone and less mitotically active trabeculations (Jeter and Cameron, 1971), expression of both ligand and receptor is further diminished (Figs. 3A–3D), and cardiac myocytes fail to express NT-3 or catalytically active trk C at later developmental stages. No expression of the truncated trk C isoform was detected in hearts examined at E9 (Fig. 3E).

Generation of Stable Truncated Trk C Viral-Producing Lines

To assess the role of NT-3 in modulating the early stages of chick heart development *in ovo*, a replication-defective retrovirus encoding truncated chick trk C was generated. Transfection of this viral vector encoding lacZ into a packaging cell line resulted in stable clones from which the virus was propagated at high titer. Expression of truncated trk C in these clones was confirmed by Western blot analysis, which demonstrated a prominent 100-kDa band corresponding to the predicted molecular size of truncated

trk C, and was absent in blots of parental packaging cells (Fig. 4A). To confirm that truncated trk C expressed by these clones was capable of binding NT-3, crosslinking analysis was performed using the radioiodinated ligand (Fig. 4B). The crosslinked product of approximately 120 kDa, composed of NT-3 (13.5 kDa) and truncated trk C (110 kDa), was detected in clones expressing the truncated trk C retrovirus, but not in parental packaging cells.

Expression of Truncated Trk C Reduces Myocyte Clone Size

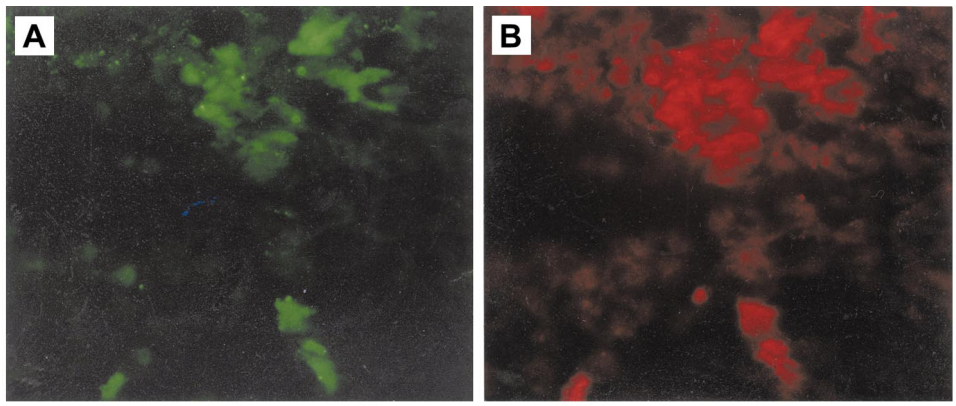
Prior studies have demonstrated that the truncated trk C receptor can inhibit NT-3 signaling when coexpressed with kinase-active trk C (Das *et al.,* 2000; Palko *et al.,* 1999). To analyze the effects of truncated trk C overexpression on cardiac morphogenesis, *in ovo* injections of 10–100 viral particles into the developing ventricle were performed at E3. Following retroviral infection and integration into the host genome, the truncated trk C transgene and the reporter gene β-galactosidase are transcribed as a dicistronic message utilizing an IRES sequence. Since the expression of β-galactosidase, translated in a 5′ cap-independent manner via the IRES element, serves as a marker for truncated trk C expression in virally infected cells, double immunofluorescence labeling of cells was performed to assess the coordinate expression of truncated trk C and β-galactosidase. Sections of E7 chick ventricle, obtained following infection at E3 with the truncated trk C/β-galactosidase-encoding virus, demonstrate that the immunolocalization of truncated trk C coincides with localization of β-galactosidase (Figs. 5A and 5B).

To assess the effects of truncated trk C expression on cardiac myocyte clone size, embryos were injected in the developing ventricular wall at E3 with retrovirus at low viral titers (10^5–10^6 virions/ml) to obtain spatially isolated colonies of cells at E7, as visualized by X-gal histochemistry. From inspection of intact hearts, clones from embryos expressing truncated trk C appeared smaller than clones from embryos infected with control lacZ virus (Figs. 6A and 6B). In analysis of serial sections from E7 ventricles infected with either control lacZ virus or truncated trk C virus, colonies generated from cells expressing control virus demonstrate labeled cells distributed from the subepicardial surface to the trabeculations (Fig. 6C). The distribution of labeled cells is consistent with formation of cone-shaped clones arising from infection of a single myocyte at E3 (Mikawa *et al.,* 1992b). Colonies generated from cells expressing truncated trk C were of smaller size (Fig. 6D). However, truncated trk C-expressing myocytes were present both in the subepicardial region of the ventricular wall and within the trabeculae, suggesting that overexpression of truncated trk C does not alter the migration of myocytes, from subepicardially located progenitors to daughter cells located nearer to the ventricular lumen. Analysis of 6 ventricular clones from control lacZ-infected embryos or 18 ventricular clones from truncated trk C-infected embryos demonstrated an average of 39 myocytes in control clones and 14 cells in truncated trk C expression clones, resulting in a 63% decrease in the size of myocyte clones in which trk C signaling was disrupted ($P < 0.001$, unpaired two-tailed t test) (Figs. 6E and 6F). Clones expressing truncated trk C in either the left or the right ventricle exhibited similar decreases in clonal size, compared to similarly positioned control lacZ-infected clones, suggesting that the effects of NT-3 signaling in the developing right or left ventricle were similar.

Expression of Truncated Trk C Reduces Cell Proliferation

The reduction in the size of truncated trk C-expressing clones suggests that NT-3-mediated trk C signaling could induce proliferation of progenitor cells or inhibit apoptosis. To determine if a dominant effect of NT-3 is to regulate cellular proliferation, BrdU incorporation was assessed in ventricles which had been infected with control or truncated trk C retrovirus at E2, E3, or E5. BrdU labeling of embryos was performed for 5 h prior to embryo harvest, at 3 to 4 days following injection as indicated (Fig. 7). The percentage of β-galactosidase-positive cells which exhibited nuclear BrdU uptake was assessed in embryos (E6) expressing either control or truncated trk C (Figs. 7A and 7B). Myocytes infected with control lacZ virus exhibited high levels of BrdU uptake at E5 (44%), with progressive decreases at E6 (39%) and E9 (25%) (Fig. 7C). These values are consistent with prior studies demonstrating rapid myocyte proliferation at the earlier developmental stages, with a gradual reduction in proliferation rate at later stages, as assessed by mean doubling times (Mima *et al.,* 1995) or tritiated thymidine labeling (Jeter and Cameron, 1971; Clark and Fischman, 1983). However, the percentage of BrdU-positive cells in truncated trk C-expressing clones was significantly reduced compared to control β-galactosidase-expressing clones at E5 (28% of trunc trk C clones vs 44% in control clones, $P < 0.005$, unpaired two-tailed t test) and at E6 (26% in trunc trk C clones vs 39% in control clones, $P < 0.01$, unpaired two-tailed t-test) (Fig. 7C). Thus, at E5 and E6, expression of truncated trk C resulted in a 34–36% reduction in BrdU incorporation compared to control viral-infected myocytes. However, by E9 no significant differences in the percentage of BrdU-positive cells were detected in truncated trk C or control-injected myocytes (22% of trunc trk C clones vs 25% of control clones), suggesting that trk C signaling regulates cardiac myocyte proliferation during the first week of embryogenesis, but has little effect at later developmental times.

As independent confirmation that truncated trk C expression reduces myocyte proliferation, PCNA immunoreactivity was quantitated in embryos infected with either control or truncated trk C virus at E3 and harvested at E6. The percentage of PCNA-positive cells in truncated trk C-expressing clones was significantly reduced compared to



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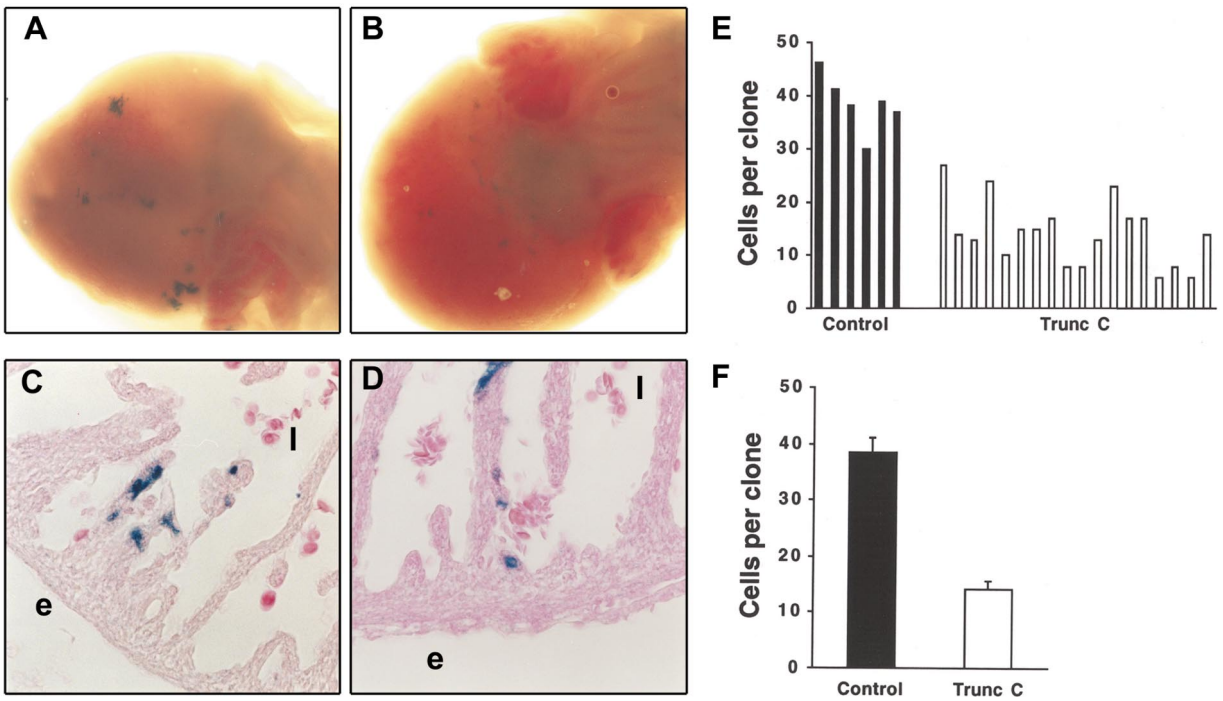

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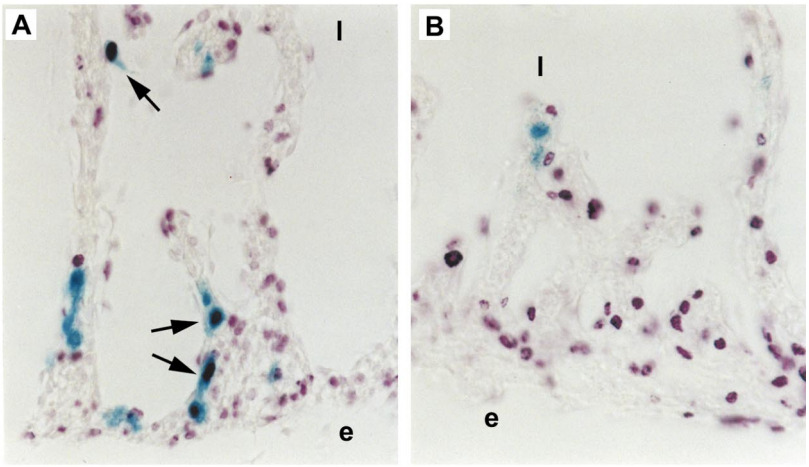
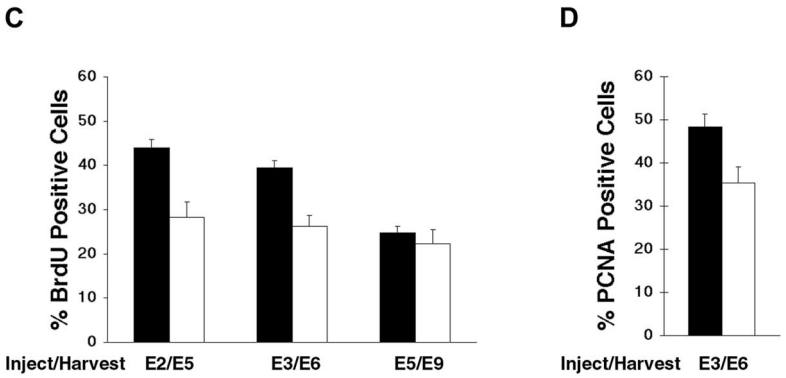
FIG. 5. Double immunolabeling of β-galactosidase and truncated trk C in chick embryonic heart. Double immunofluorescence labeling was performed to document the coexpression of truncated trk C and β-galactosidase in chick hearts injected with the retroviral vector encoding both genes. Embryos were injected at E3 and incubated until sacrifice at E7. Immunohistochemistry was performed using mouse anti-β-galactosidase antibody with a fluorescein-conjugated secondary antibody (A) and a rabbit anti-truncated trk C antibody with a Texas red-conjugated secondary antibody (B). Note the colocalization of both β-galactosidase and truncated trk C in virally infected myocytes. Sections stained with only one primary and secondary antibody exhibited no signal in the other channel, indicating the absence of cross-contamination of signal. (A and B, original magnification 400×).

FIG. 6. Expression of truncated trk C results in reduced clone size in E7 chick embryonic hearts by whole-mount and histological analyses. Chick embryos are injected with either the control virus (vector encoding β-galactosidase gene alone, A and C) or the trunc trk C virus (vector encoding both β-galactosidase and truncated trk C genes, B and D) at E3 and harvested at E7. Following fixation in paraformaldehyde, hearts were treated with X-gal to generate a blue precipitate in β-galactosidase-expressing cells. The hearts were subsequently sectioned, counterstained with eosin, and analyzed microscopically. The size of each clone was determined by counting the number of β-gal-positive cells in serial sections and tabulated as individual clone counts (E) or as average clone size (F). Overexpression of truncated trk C results in a 63% reduction in average clone size compared to control clones. e, epicardium; l, lumen of ventricle. (A and B, original magnification 35×; C and D, 400×)





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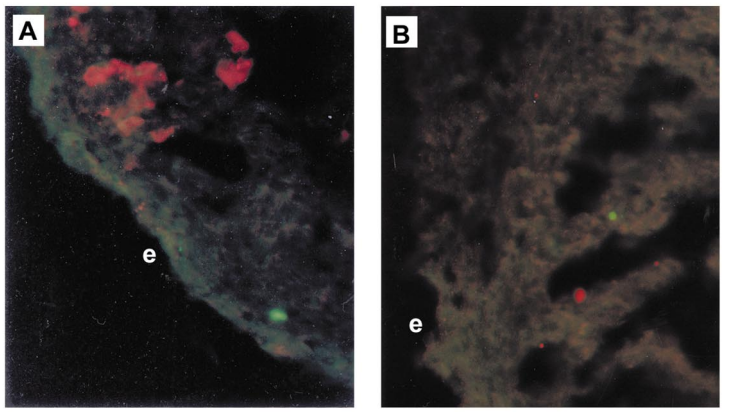

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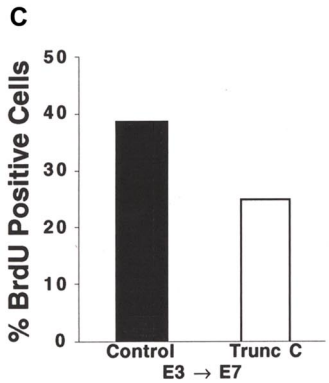
TUNEL (+) & β-Gal (+) / β-Gal (+) = 1/164



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control β-galactosidase-expressing clones at E6 (35% of trunc trk C clones vs 48% of control clones, $P < 0.02$, unpaired two-tailed *t* test) (Fig. 7D).

The reduction in BrdU incorporation and reduction in PCNA immunoreactivity upon truncated trk C expression at both E5 and E6 suggest that cumulative antiproliferative effects of truncated trk C expression can result in the 63% reduction in myocyte clone size observed at E7. However, to formally exclude an apoptotic effect upon truncated trk C expression, TUNEL analysis was performed on embryonic hearts at E5, E6, or E7 following retroviral infection with lacZ or truncated trk C virus at E3. Embryos had additionally been pulse-labeled with BrdU for 5 h prior to embryo harvest, so that BrdU incorporation and TUNEL analysis could be directly compared. Consistent with the results obtained at E5 and E6, truncated trk C-expressing myocytes demonstrated reduced BrdU incorporation compared to control viral-infected myocytes at E7 (39% BrdU incorporation in control myocytes, 25% BrdU incorporation in truncated trk C myocytes, yielding a 35% reduction in BrdU incorporation) (Fig. 8C). However, upon concomitant immunofluorescence localization of β-galactosidase and TUNEL reactivity, less than 1% of the β-galactosidase-expressing cells exhibited TUNEL positivity in embryos infected with either control or truncated trk C virus (Figs. 8A and 8B). Indeed, TUNEL analysis at E5 or E6 of embryos injected with either CXL or truncated trk C virus also yielded less that 1% of the β-galactosidase-expressing cells exhibiting TUNEL reactivity (the ratio of TUNEL/β-gal double-positive cells to β-gal-positive cells at E5 was 1/124 in control virus-infected hearts, 1/198 in trunc trk C-infected hearts and at E6 was 5/568 in control virus-infected hearts and 3/380 in trunc trk C-infected hearts). These results suggest that the dominant action of NT-3 on embryonic cardiac myocytes is to induce cell proliferation.

DISCUSSION

NT-3 Is a Physiological Mitogen for Cardiac Myocytes

By utilizing retroviral gene delivery to inhibit NT-3-dependent trk C signaling, our studies establish that NT-3 is a potent physiologic mitogen for embryonic ventricular cardiac myocytes. These studies identify NT-3 as one of a small number of growth factors regulating cardiac myocyte proliferation during early development. Indeed, only FGF receptor signaling, most likely initiated by FGF-2 and FGF-4, has been demonstrated to induce cardiac myocyte proliferation during the rapid phase of ventricular wall growth in the first week of chick development (Zhu *et al.*, 1996; Mima *et al.*, 1995). The observed inability to completely suppress myocyte proliferation during cardiac chamber formation by inhibiting either FGF receptor signaling (Mima *et al.*, 1995) or trk C receptor signaling suggests that both growth factors may act at a similar developmental stage to regulate myocyte proliferation.

FIG. 7. Expression of truncated trk C results in reduced BrdU incorporation in E5 and E6, but not in E9, chick hearts. Embryos were injected with the control or truncated trk C-expressing virus at E2, E3, or E5 and subjected to BrdU pulse for 5–6 h prior to sacrifice at E5, E6, or E9, respectively. Following harvesting of chick hearts and development in X-gal, immunohistochemical analysis of BrdU incorporation was performed on heart sections (A, hearts injected with CXL at E3 and harvested at E6; B, hearts injected with trunc trk C at E3 and harvested at E6). (C) Sections were analyzed for the percentage of β-galactosidase-expressing cells which exhibited nuclear BrdU uptake, with intense purple staining within the β-galactosidase-positive blue cell (arrow). 150 to 400 β-galactosidase-positive cells were analyzed in each embryo, and the percentage of β-galactosidase-positive cells which exhibited nuclear BrdU immunoreactivity was determined for each embryo. For E2 to E5 analysis, five CXL- and five truncated trk C-injected hearts were examined. For E3 to E6 and for E5 to E9 analysis, three CXL- and three truncated trk C-injected hearts were examined. The results obtained with each embryo were averaged for each time point, with the means and standard errors of the mean represented graphically, with the filled columns representing data obtained with CXL-infected hearts and the open columns representing data obtained with trunc trk C-infected hearts. (D) Sections were analyzed for the percentage of β-galactosidase-expressing cells which exhibited PCNA immunoreactivity, with intense purple staining within the β-galactosidase-positive blue cell. 150 to 400 β-galactosidase-positive cells were analyzed in each embryo, and the percentage of β-galactosidase-positive cells which exhibited nuclear PCNA immunoreactivity was determined for each embryo. Results obtained with each embryo were averaged, with the means and standard errors of the mean represented graphically, with filled columns representing data obtained with control virus, open columns with trunc trk C virus. e, epicardium; l, lumen of ventricle. (A and B, original magnification 400×).

FIG. 8. Expression of truncated trk C does not induce cardiac myocyte apoptosis in E7 chick hearts. Embryos were injected with control virus or trunc trk C-expressing virus at E3 and were pulsed with BrdU for 5 h prior to harvesting at E7. (A and B) TUNEL reactivity and β-galactosidase immunolocalization were performed concomitantly on heart sections using fluoresceinated dUTPs to detect TUNEL-positive cells and rhodamine-conjugated antiserum to detect β-galactosidase-expressing cells. The ratio of TUNEL/β-galactosidase double-positive cells to β-galactosidase-positive cells is noted below each image and is representative of results obtained in two embryos examined per condition. (A) Embryos infected with control virus; (B) embryos infected with trunc trk C virus. (C) Sections were analyzed for the percentage of β-galactosidase-expressing cells which exhibited nuclear BrdU uptake, as described in Fig. 7. More than 500 β-galactosidase-positive cells were analyzed per condition. The filled columns represent data obtained with CXL-infected hearts, and the open columns represent data obtained with trunc trk C-infected hearts. e, epicardium. (A and B, original magnification 400×).

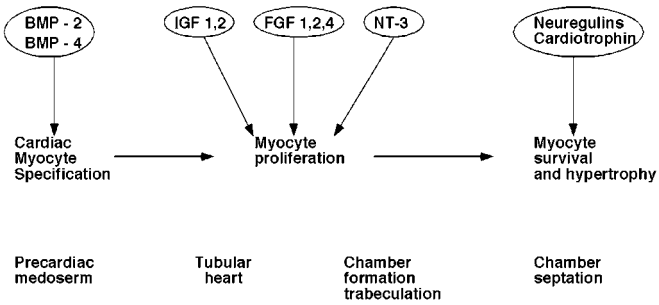

FIG. 9. Schematic representation of growth factors regulating cardiac myocyte commitment, proliferation, and survival at distinctive developmental stages. This drawing reflects findings described by Ladd *et al.* (1998); Antin *et al.* (1996); Zhu *et al.* (1996); Mima *et al.* (1995), this study, Zhao *et al.* (1998), and Wollert and Chien (1997).

Indeed, a stage-by-stage comparison of NT-3:trk C localization (this study) with FGF-1, -4:FGFR1 localization in developing chick hearts (Consigli and Joseph-Silverstein, 1991; Sugi *et al.*, 1995; Zhu *et al.*, 1996) suggests that both NT-3 and FGFs may act upon myocytes of the looping heart (stages 14–17, E2–2.5) to regulate proliferation. However, by stage 24–32 (E4.5–7.5) when ventricular trabeculation is well established, the patterns of expression of the two ligand:receptor systems are distinct and reciprocal; NT-3/trk C expression is prominent within the trabeculae, and diminishing in the compact zone, whereas FGF/FGFR1 expression is reduced in the trabeculae, but more prominent in the compact zone. These observations suggest that both FGFs and NT-3 may regulate mitogenesis from E2 to E5 during the transition from a looping heart to a heart with established trabeculations. From E4.5 to E7.5, FGF signaling may act to promote the continued proliferation of myocytes within the developing compact zone, while NT-3 regulates the significant proliferation of myocytes within the developing trabeculae, where continued BrdU uptake is readily detectable (Fig. 7). Given the temporally overlapping expression of FGFR and trk C receptor in ventricular myocytes, it will be important in future studies to determine if these growth factors act in a complementary manner in the looping heart and in a spatially restricted manner upon cardiac myocytes of the trabecular wall.

Other growth factors which have been postulated to regulate myocyte proliferation include the insulin-like growth factors, neuregulin, and cardiotrophin-1. Prior *in vitro* studies have documented that insulin-like growth factors (IGF-1 and IGF-2) can promote proliferation of precardiac mesodermal cells at stages prior to tubular heart formation (Antin *et al.*, 1996). At later stages of cardiac development when myocyte proliferation has slowed, neuregulin and cardiotrophin-1 exert modest proliferative effects *in vitro*, but more prominent survival-promoting and hypertrophic responses (Ford *et al.*, 1999; Kuwahara *et al.*, 1999; Zhao *et al.*, 1998). Thus the sequential regulation of cardiac myocyte commitment, proliferation, and survival/hypertrophy may reflect myocyte responsivity to several classes of peptide growth factors, each utilized at a distinctive developmental stage (Fig. 9).

That NT-3 may serve as a mitogen for cardiac myocytes in the looping heart, and during the processes of trabeculation and chamber formation, is supported by evidence of the early localization of this neurotrophin and receptor in the developing heart. Although NT-3 mRNA is detectable in early avian embryos (HH stage 6), when heart precursors reside in the lateral mesodermal plate, no tissue localization has been performed to date (Zhang *et al.*, 1996). Our prior, limited studies in the mouse have documented trk C protein expression by cardiac myocytes at murine embryonic day 9.5, which is similar with regard to cardiac development to avian embryonic day 3 to 4 (Donovan *et al.*, 1996). This immunohistochemical localization of trk C to the ventricles is consistent with earlier *in situ* analysis in the developing mouse embryo (Tessarollo *et al.*, 1993). However, the inability to detect NT-3 and trk C mRNA at later stages of heart development in rats (E11–18) (Hiltunen, 1996) is consistent with our observations of decreased trk C expression during later stages of trabecular formation at avian E9. This dynamic regulation of both ligand and receptor during cardiac development and their restricted expression by cardiac myocytes, but not by epicardial or endocardial cells or cells within the endocardial cushion, suggest that NT-3 and trk C genes may be induced by well-characterized cardiac transcriptional regulators (as reviewed by Fishman and Chien, 1997), questions amenable to genetic dissection.

The colocalization of both ligand and receptor to cardiac myocytes suggests an autocrine or local paracrine action of this growth factor:receptor system. Our finding that locally derived NT-3 acts directly on a population of rapidly dividing cardiac myocytes is similar to that observed with the FGFs, which colocalize with FGFR in cardiac myocytes during chamber formation. This contrasts with the more complicated expression patterns of growth factors acting at later developmental stages to regulate cardiac myocyte proliferation or hypertrophy. Indeed, the expression of cardiotrophin-1 by cardiac fibroblasts (Kuwahara *et al.*, 1999) and neuregulins by the endocardium and microvascular endothelial cells (Hertig *et al.*, 1999; Zhao *et al.*, 1998) suggests that growth factors acting at later developmental stages are subject to more complex regulatory inputs, befitting a myocardium which must respond to hypoxic or hypertensive insults.

Pleiotrophic Roles of NT-3 during Development

The biological actions of trk C on cardiac myocyte proliferation are likely to underlie the defects on cardiac ventricular septation and possibly those of ventricular wall thinning observed in the NT-3- and trk C-deficient mice (Donovan *et al.*, 1996; Tessarollo *et al.*, 1997). However, the

trk C and NT-3 null mutant mice also exhibit defects in valvulogenesis, outflow tract septation and rotation, and closure of ductus arteriosus, malformations which model, in part, the human congenital condition of Tetralogy of Fallot. Although the molecular pathogenesis of these defects in humans is still not defined, abnormalities in cardiac neural crest as well as cardiac myocyte development have been postulated to contribute to this complex phenotype (Kirby, 1999). Thus it will be important in future studies to assess whether NT-3-mediated trk C signaling could impart additional actions upon cells of cardiac neural crest origin to contribute to the observed abnormalities in valve and outflow tract formation. Indeed, expression studies confirm that trk C is expressed by trunk neural crest, and NT-3 can promote the proliferation and differentiation of neural crest *in vitro* and *in vivo* (Chalazonitis *et al.*, 1998; Henion, 1995; Kalcheim *et al.*, 1992). However, further studies to directly inhibit or augment NT-3 actions on this cell population will be required to evaluate the potential roles of NT-3 on cardiac neural crest survival or migration.

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